ORIGINAL ARTICLE

Enteric Fever in a Cross-section of Patients in Karachi: Current Correlation of Positive Blood Cultures with the Widal Agglutination and the Typhidot Immunoassay Tests

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ABSTRACT

Objectives: A lab-based scrutiny to assess the correlation of current blood culture Salmonella isolates as the gold standard with matched Widal agglutinin titers and the EIA-based Typhidot immunoassay antibodies.
Methods: 2,704 blood specimens were drawn for culture and Widal test in a private clinical lab in Karachi City from subjects presenting with clinical signs of Enteric fever during April 2012 and March 2013. Of these, 1,497 yielded Salmonella isolates; sera of these patients that were not accompanied with requests for Typhidot were also subjected to dot immunoassay with informed consent solicited at the time of bleeding. All sera were stored at 4^0C until screened.
Results: Blood from 802 males (53.6%) and 695 females (46.4%) yielded 61.85% *S typhi* (*n*=926), 31.26% *S. paratyphi-A* (*n*=468) and 6.88% *S. paratyphi-B* (*n*=103) isolates. Widal agglutinins were detected in 473 (31.5%) of the sera of these subjects. 'H' titers of 1:80 (*n*=264: 17.6%) without detectable 'O' antibodies were seen more frequently in children's sera (*n*=112; 7.4%). Widal 'H' with 'O' agglutinins were recorded in 209 (13.9%) of corresponding positive blood cultures, of which 104 (6.9%) yielded titers of 1:320 or more. A total of 1,024 sera (58.4%) lacked detectable Widal antibodies. The Typhidot immunoglobulin dots, negative in 856 sera (57.1%), were detected in 641 specimens (42.8%). IgM dots (*n*=314: 20.9%) without IgG were more commonly seen, as were IgM with IgG (*n*=296: 19.7%). Also noted were IgG dots with no detectable IgM in 22 sera (1.47%), and IgM dots with no IgG in specimens that yielded *S. paratyphi*-A (*n*=8) and one *S. paratyphi*-B isolate.
Conclusion: *S. paratyphi-A* has increased in frequency during the last decade, possibly suggesting incomplete protective coverage employing monovalent vaccine. Widal agglutinins and Typhidot antibodies were detectable in 31.5% and 42.8% respectively in the sera of patients whose blood grew *Salmonellae*. The Widal can be misconstrued due to possible "background" agglutinins in the unvaccinated, and the dot EIA immunoassay is restricted in specificity for only *S. typhi*. An ICT-based gauge of the three Salmonella serotypes is desirable.

Key words: Enteric fever, Widal, Typhidot, Blood Cultures, Salmonella.

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INTRODUCTION

Enteric fever is said to affect 21.6 million people annually and kill an estimated 600,000 worldwide each year,[1] but is mainly prevalent in developing countries with 80% cases occurring in Pakistan, Bangladesh, India, Indonesia, China, Laos, Nepal and Vietnam.[2-7] Within these countries typhoid is more often seen in below par developed areas due to poor sanitation, contaminated food and drinking water, illiteracy and low socioeconomic status.[4]

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Pakistan is a hyper-endemic area for the infection, and although the precise incidence is variable, enteric fever is possibly the fourth most common cause of death in the country. Once a disease of the summer, it is now prevalent throughout the year in cities like Karachi, more so following the monsoon rain.[2,6,7] Moreover, the problem has been further aggravated by the emergence of Quinolone-resistant salmonella strains.[8-11]

Therefore, rapid and precise diagnosis, along with pertinent effective antibacterial therapy is important in reducing morbidity and mortality from complications like intestinal perforation and hemorrhage. The Widal agglutination test is most commonly used for the retrospective or presumptive diagnosis of clinically suspected enteric fever patients. This historical test has continued into the 21st century ever since Georges Widal (1896) showed that patient's sera would clump

typhoid or paratyphoid bacilli if it contained antibodies against the organisms' lipopolysaccharide 'O' and/or protein flagellar 'H' antigens.[12-21] It is also used widely in Pakistan due to its low cost, rapidity and simplicity and since the facility of the gold standard blood culture is still not available in many clinical labs even in a mega-city like Karachi. An alternative test, the EIA-based Typhidot immunoassay which was introduced in Karachi in 1996 is designed to specifically detect IgM and/or IgG in patient's serum against an immunogenic 50kDa outer-membrane protein claimed to be specific for *Salmonella enterica serovar typhi* (*S.* typhi) on nitrocellulose strips.[22,23] The Widal, however, remains in common use if there is a strong clinical suspicion of enteric fever, and when suggestive, also aids in ruling out a number of other febrile diseases that mimic typhoid in early symptoms, like dengue and malaria.

This study, aside from determining the current prevalence of the three major etiological serotypes that cause enteric fever in our environment, which has a bearing on the outcome of pertinent vaccines in use, and judging from reports in the literature, is apparently a lone attempt designed to evaluate the incidence of Widal agglutinin titers along with Typhidot IgM and IgG antibodies, if any, in patients' blood which grow salmonella in culture. This would not only provide an insight on the antibody levels in patients with culture-confirmed enteric fever, but advocate whether the antibodies, although present in their circulation, ostensibly for some reason do not interfere with the organisms' infective progress.

METHODOLOGY

Blood samples from a total of 2704 patients with suspected enteric fever referred to a local private diagnostic lab with branches in key areas of Karachi City between April 2012 and March 2013 were collected on physicians' request for culture and the Widal agglutination test. The subjects, aged 2 months to 79 years, were divided into 8 groups on the basis of age.

For blood culture, approximately 5-10ml of venous blood was collected by venipuncture with aseptic measures from adults, and 2-3ml from children. Each specimen was promptly inoculated directly into the Oxoid Signal blood culture system (Basingstoke, UK) containing a unique broth medium which enables a wide range of aerobes and anaerobes to be cultured in a single bottle. Each charged bottle was incubated at 37^0C for one hour and then the Signal growth indicator chamber device was inserted through the capped neck of the bottle. The sealed system was next placed on an orbital shaker for the first 24 hours of the 7-day incubation period, in accordance with the manufacturer's instructions, and checked regularly for visual indication of a positive sample because of broth/blood mixture being forced into the upper chamber by gases formed during growth and replication of the organism in the system. With each positive result, samples were removed directly from the indicator chamber for further testing by sub-culturing on blood agar and EMB medium (Oxoid, UK), and the isolate identified using API 20 E cupules. A total of 1,497 cultures yielded positive growth of Salmonella isolates, which were further subjected to serological confirmation using specific antisera (Difco, USA).

An extra ml of blood was also bled for serum from each of the subjects for the two serological tests. The conventional Widal tube agglutination test was performed using the Widal Antigen (Spectrum Diagnostics, Germany) for the determination of anti-Salmonella antibodies. A 0.4ml aliquot of each of two-fold serially diluted patients serum (1:20 to 1:320) in 0.9% normal saline were tested by adding an equal volume of antigen suspension. A negative saline control was included with each batch. The charged tubes in a metal rack were kept immersed in a water-bath at 37^0C for 1hr. A dilution ratio of 1:80 or greater showing agglutination was taken as the titer possibly indicative of typhoid or paratyphoid fever.

For the Typhidot, the presence of IgM and IgG antibodies was detected by incubating nitrocellulose strips (Reszon Diagnostics, Selangor, Malaysia) dotted with the specific antigen with 0.5ml of subject's serum and of control sera. The antigen-antibody complex was visualized directly by incubating the strips simultaneously with peroxidase-conjugated anti-human IgM and IgG. Upon addition of chromogenic substrate, positive tests were interpreted according to the intensity of resultant blue color compared with positive control in a total assay time of 3 hours.

Descriptive statistics of SPSS 17 and Microsoft excel 2010 were used to analyze data.

RESULTS

The total number of patients (n=2,704) referred for blood culture and the Widal test on the basis of clinical symptoms included 1,539 males and 1,165 females. Blood drawn from 55.3% of these subjects (n=1,497) yielded *Salmonellae* within 3-5 days of culture. The isolates were *S. typhi* (n=926: 61.85%), *S. paratyphi-A* (n=468: 31.26%), and *S. paratyphi-B* (n=103: 6.88%) illustrated in Fig. 1.1.

Among the 1,497 blood specimens that grew the three Salmonella serotypes were 802 males (53.6%) and 695 females (46.4%) aged 2 months to 79 years (Fig. 1.2). A total of 380 children between the ages of 2m and 16

years yielded *Salmonellae*, while the 1,117 adults that also grew enteric fever isolates included 19 aged 17 to 19 years. The numbers of positive cultures were inversely proportional to age of patient, decreasing from 328 in age group 3 (Fig. 1.3) to 08 in the elderly (group 8). The maximum number of positive cases presented at the age of 20-29 years (*n*=328). The mean age was 23.21 years.

Fig. 1.1: Current Prevalence of Salmonella serotypes

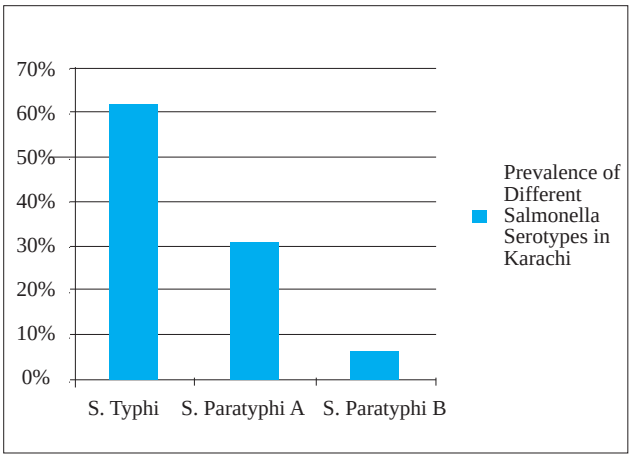
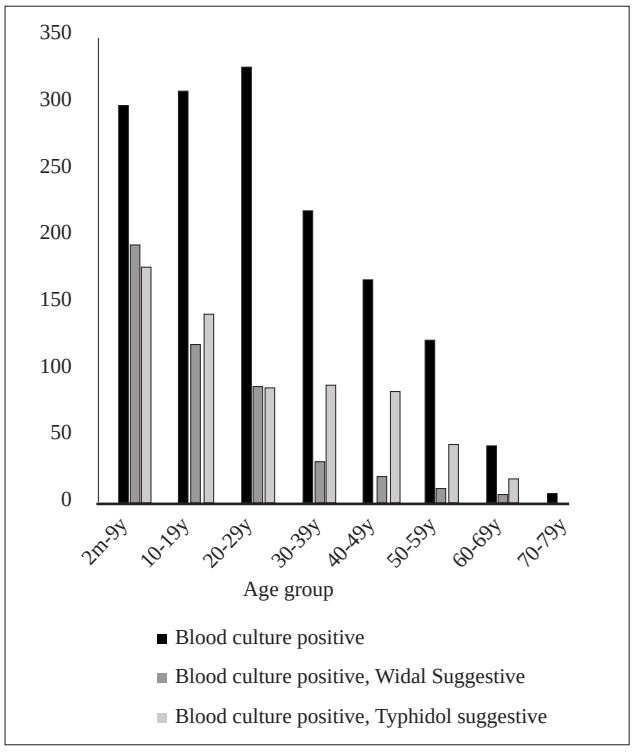


Fig. 1.3: Positive Blood cultures with suggestive Enteric fever, Widal and Typhidot in different age groups.



Results of the assay of Widal agglutinins and Typhidot antibodies in blood specimens that yielded *Salmonellae* are presented in Table 1. Widal agglutinins were detected in 473 (31.5%) of the sera of these patients. 'H' titers of 1:80 (*n*=264; 17.6%) without noticeable 'O' antibodies were seen, but more frequently in children's sera (*n*=112; 7.4%). Widal 'H' along with 'O' agglutinins were recorded in 209 (13.9%) of corresponding blood cultures, of which 104 (6.9%) yielded titers of 1:320 or more. A total of 1,024 sera lacked demonstrable Widal antibodies.

The Typhidot was negative in 856 sera (57.1%). The test dots were, nevertheless, detected in 641 specimens (42.8%). IgM dots (n=314: 20.9%) without IgG were mostly seen, as were both IgM with IgG dots (*n*=296: 19.7%). Also noted were IgG dots with no determinate IgM in 22 sera (1.47%), and IgM with no signs of IgG in blood specimens that yielded *S. paratyphi-A* (*n*=8) and one *S. paratyphi-B* isolate.

Table 1: Positive Blood cultures with suggestive Enteric fever, Widal and Typhidot in different age groups.

Age Groups	Clinically suspected enteric fever cases (n)	Blood culture positive (n, %)	Blood culture positive, Widal suggestive (n, %)	Blood culture positive, Typhidot suggestive (n, %)
2m-9y	692	299 (19.9 %)	193 (12.8%)	178 (11.8%)
10-19y	612	310 (20.7%)	120 (8.0)	141 (9.4%)
20-29y	624	328 (21.7%)	88 (5.8%)	87 (5.8%)
30-39y	314	219 (14.6%)	32 (2.1%)	89 (5.9%)
40-49y	215	168 (11.2%)	21 (1.4%)	84 (5.6%)
50-59y	160	122 (6.7%)	12 (0.8%)	44 (2.9%)
60-69y	68	43 (2.8%)	7 (0.4%)	18 (1.2%)
70-79y	19	8 (0.5%)	-	-
Total cases	2704	1497 (55.3%)	473 (31.5%)	641 (42.8%)

DISCUSSION

Typhoid fever continues to be a major endemic health problem in Pakistan. Rapid population growth, increased urbanization, inadequate human waste disposal, limited safe water supply and overburdened heath care systems have all made the disease control difficult. The main causative agent of enteric fever remains *S. typhi* (61.85%), which is in accordance with previous studies conducted in Pakistan[2,3,6,7,10] and other countries.[4,5,11,13,14,16-18,24] However, compared to previous reports in which *S. paratyphoid-B* fever had little occurrence,[1,4,5] the incidence of this serotype causing the illness is increasing, and is 6.88% among our subjects.. Even more remarkable is the growing manifestation of *S. paratyphi-A* in enteric fever cases reported in 2004 by Anjum et al[8] and observed during 2008-2010 by Abdullah et al.[25] Currently, in our hands the incidence of this serotype is calculated to be 31.26% in a cross-section of patients in Karachi, which is considered of import The relative frequency of paratyphoid fever, previously considered to occur less often and a cause of a more benign disease than typhoid

fever, has indeed also increased during the last decade in China, India, Nepal, Thailand and the United States[1,4,11,15,22] possibly suggesting incomplete protective coverage due to the replacement of bivalent TA vaccine with monovalent vaccines effective only against *S. typhi.*

Males were more prone to be infected than females, which is in agreement with previous studies conducted [7,25] and advocates that this trend has not changed, suggesting that males in their lifestyle were more prone to be exposed to Salmonella infection.

The gold standard for diagnosis of typhoid and paratyphoid fever is isolation of *S. typhi* and *S. paratyphi* from the body fluids of patients, especially blood and bone marrow. However, blood cultures fail to detect 10 -70% of patients with enteric fever due to several reasons: the inadequate amount of blood sampled, relying most often on a single specimen, the phase of the disease in the patient at the time of bleeding, and antibiotic usage commenced prior to sampling. Also, culture usually requires 3-5 days for results, hence encouraging commencement of empirical prescription.[12] In previous studies it has been shown that a high proportion of the TYP-CN patients were typhoid but were missed by culture.[2] Serological information is thus relied upon, and the most commonly used conventional method remains the classical Widal agglutination test with its advantages of low cost and easy conductance.[9]

In the original format, the Widal required acute- and convalescent-phase serum samples taken approximately 10 days apart, but more recently, the test has been adapted for use with a single, acute phase serum sample.[12] The present study likewise reveals that a single acute phase Widal test when suggestive can be a convenient diagnostic tool for typhoid and paratyphoid fever. Raised titers (1:160) of especially 'O' with 'H' antibodies have always been considered as confirmatory, but in our studies we observed 'H' titers of 1:80, and occasionally 1:160, without detectable 'O' agglutinins, in 17.6% blood-culture proven cases, especially in children and adolescents, indicating that this result is also significant and generally corresponds with the early phase of fever. However, the Widal ('H' and/or 'O' titers) was suggestive in only 31.5% of overall matching positive blood cultures, indicating a low sensitivity.

The Typhidot immunoglobulin dots, on the other hand, were detected in 42.8% of the sera screened, with IgM dots without IgG seen more often in patients who presented with 1-3 days of fever. This observation was reasonable since IgM is the first immunoglobulin class produced against foreign antigens, followed by IgG. IgM accompanied IgG in blood drawn between 3-6 days of fever in 19.7% of sera, which, along with instances in which IgM alone was detected, accounted for a sensitivity of 96%. In contrast, the Widal was positive in only 31.5% of culture-proven cases, and with titers of especially 'H' seen not earlier than the 5th day of fever.

The Typhidot yielded three additional anomalies that need explanation. Occasional patients (*n*=5) whose sera gave only detectable IgM later reported becoming afrebrile within a day or two after providing the blood sample, without having commenced antibiotherapy, raising the query of whether 'subclinical' infection can occur in typhoid fever, as is known in several other diseases like diphtheria, polio and whooping cough. Secondly, IgG dots alone, without detectable IgM were interestingly noted in 22 cases during the study; this could possibly occur following vaccination, in the 'late' stage of infection, during convalescence or relapse, or in carriers. Thirdly, eight *S. paratyphi-A*, and one *S. paratyphi-B* isolate were grown in blood cultures from nine subjects whose Typhidot results were positive, suggesting occasional cross-reaction between *S. typhi* and other *Salmonellae*, and compromising the specificity of the Typhidot.

Finally, this interesting observation n our study is underlined: *Salmonellae* were grown from patient's blood specimens that also contained varying amounts of specific Widal agglutinins and Typhidot immunoglobulins, suggesting that these 'antibodies' did not prevent the organism from continuing on its disease-producing path. Whether this indicates a paucity of cooperative protective immune factors like complement C3 or the like in Enteric fever patients, is a conjecture worth exploring.

Though culture (blood, bone marrow) remains the gold standard, a rapid, definitive lab assay for typhoid is evidently needed, since presentation can be ill-defined and vary significantly in patients, especially in children. The Widal can be misconstrued due to possible "background" agglutinins in the unvaccinated, and the dot immunoassay is restricted in specificity for only *S. typhi*. Hence an ICT-based gauge of the three commonly encountered Salmonella serotypes in our country is desirable.

CONCLUSION

Enteric fever is largely prevalent in Pakistan and is on the rise in the metropolis affecting mostly school-aged children and young adults. *Salmonella typhi* remains as the main causative agent of enteric fever, but the incidence of *Salmonella paratyphi A* has significantly increased during the last decade. Since a high proportion

of population in Pakistan are non-affording, the Widal test continues as an inexpensive and easily conducted test for the presumptive diagnosis of enteric fever, and when positive, can indicate any of the three Salmonella serotypes. The Typhidot is expensive, requires expertise, is restricted in specificity for detecting only *S. typhi*, but has the advantage of providing IgM dots as early as 1-3 days of typhoid fever.

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